EP 20844 (5)

PCT WORLD INTELLE(I

INTERNATIONAL APPLICATION PUBLISHI

WO 9602257A1

(51) International Patent Classification 6 :
A61K 31/70, C07H 21/02, 21/04, C12N 15/00, 5/00, C12P 19/34, C12Q 1/68

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/US95/08781

(22) International Filing Date: 18 July 1995 (18.07.95)

(30) Priority Data:
08/276,330 18 July 1994 (18.07.94) US

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(81) Designated States: AU, CA, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTISENSE OLIGONUCLEOTIDES OF PLEIOTROPHIN

(57) Abstract

Antisense oligonucleotides that hybridize to segments of the mRNA corresponding to the cDNA for pleiotrophin inhibit synthesis of pleiotrophin *in vitro* and *in vivo*. Pharmaceutical compositions containing these oligonucleotides as the active ingredients also are disclosed.

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ANTISENSE OLIGONUCLEOTIDES OF PLEIOTROPHIN

BACKGROUND OF THE INVENTION

Polypeptide growth factors have been shown to play important physiological roles in the timely development of tissues during embryonal and neonatal growth and, therefore, their expression is tightly regulated. Conversely, polypeptide growth factor gene expression is deregulated in tumor cell lines, as well as in solid tumors, and the activity of the corresponding growth factors appears to contribute significantly to autocrine and paracrine stimuli. Cross and Dexter, *Cell* **64**:271 (1991).

Pleiotrophin (PTN) is an 18 kD heparin binding protein originally purified as a weak mitogen from bovine uterus and as a neurite outgrowth promoter from neonatal rat brain. Milner *et al.*, *Biochem. Biophys. Res. Commun.* **165**:1096-1103 (1989); Rauvala, *EMBO J.* **8**:2933-2941 (1989); Li *et al.*, *Science* **250**:1690-1694 (1990). PTN appears to belong to a family of heparin binding growth factors. Lai *et al.*, *Biochem. Biophys. Res. Commun.* **187**:1113-1121 (1992). The cDNA's for human, bovine and rat PTN's have been cloned and sequenced and shown to exhibit sequence identity with a retinoic acid-induced differentiation factor and retinoic acid-induced heparin binding protein from chicken embryo. Li *et al.* (1990); Kadomatsu *et al.*, *Biochem. Biophys. Res. Commun.* **151**:1312-1318 (1988); Tomomura *et al.*, *J. Biol. Chem.* **265**:10765-10770 (1990); Vrios *et al.*, *Biochem. Biophys. Res. Commun.* **175**:617-624 (1991).

Preliminary studies suggest that PTN transcripts are expressed in a restricted pattern within tissue and are highly regulated during murine development. PTN and the closely related midkine (MK) proteins appear to play a role during development of the neuroectoderm, and the physiologic expression of the genes in the adult occurs only in very restricted areas of the nervous system. Böhlen and Kovesdi, *Prog. Growth Factor Res.*, **3**:143-157 (1991).

PTN also has been linked to cancer formation. For example, expression of PTN is elevated in melanomas that are highly vascularized, and PTN supports the growth of SW13 cells in soft agar. Wellstein *et al.*, *J. Biol. Chem.* 267:2582-2587 (1992). PTN expression can induce tumors to grow in nude mice, and high levels of PTN mRNA are detected in tissue samples from human breast cancers. Fang *et al.*, *J. Biol. Chem.* 267 25889-25897 (1992). In the same study, about one-fourth of tumor cell lines tested showed expression of PTN, as measured by RNase protection assays. Carcinogen-induced tumors in rat mammary tissue also scored positive for PTN expression. Fang *et al.* (1992). In recent studies using PTN-targeted hammerhead ribozyme constructs, when production of PTN was quenched in WM852 human melanoma cells, soft agar colony formation was inhibited and tumorigenesis in mice was prevented. Czubayko *et al.*, *J. Biol. Chem.* in press (1994).

Other reports, however, provide conflicting data about the correlation between high PTN levels and neoplasticity. For example, Garver *et al.*, *Am. J. Respir. Cell Mol. Biol.* 9:463-466 (1993), found significantly higher PTN expression in healthy lung tissue than in malignant lung tissue. Similarly, human carcinoma tissue PTN mRNA was shown to be barely detectable in many samples, except for significant levels of expression found in PA-1 teratocarcinoma cells and in some surgical specimens of Wilms' tumor. Tsutsui *et al.*, *Cancer Res.* 53:1281-1285 (1993). Thus it remains unclear whether inhibition of PTN expression is likely to be effective in inhibiting tumor formation and growth.

There are currently no methods known for inhibiting the action of pleiotrophin in cells and determining the resulting effects on cell growth. It is apparent therefore that techniques to establish the role of pleiotrophin in tumorigenesis are greatly to be desired. In particular, it is greatly desirable to provide compositions and methods to inhibit the cellular

effects of pleiotrophin which are highly specific, and which can inhibit or prevent the pathological growth of tissue such as that found in neoplastic and dysplastic disease.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a means to inhibit pleiotrophin expression in cells.

It is also an object of this invention to provide a means of cancer therapy by inhibiting pleiotrophin expression.

It is a further object of this invention to provide compositions that inhibit the expression of pleiotrophin.

It is yet a further object of this invention to provide antisense oligonucleotides which inhibit pleiotrophin expression by controlling translation of the mRNA corresponding to the pleiotrophin gene.

In fulfilling these objects, there is provided a method for inhibiting expression of PTN in a cell by introducing an oligonucleotide that is capable of hybridizing to the single-stranded mRNA encoding human pleiotrophin.

In a preferred embodiment, the PTN is a human PTN.

In a more preferred embodiment, the oligonucleotide is contained in a liposome.

There also is provided a set of antisense oligonucleotides which, when introduced into a cell expressing PTN, inhibit PTN expression.

In another embodiment, there is provided a composition comprising at least one antisense oligonucleotide that, when introduced into a host cell, binds to a segment of a single-stranded mRNA transcribed from a pleiotrophin gene, and that inhibits pleiotrophin synthesis in said cell.

In another embodiemt, there is provided a pharmaceutically useful preparation comprising at least

one PTN antisense oligonucleotide in a pharmaceutically acceptable sterile vehicle.

In yet another embodiment, there is provided a method for treating a pathological growth of tissue, comprising the step of inhibiting expression of a pleiotrophin gene.

In a preferred embodiment, the pathological growth is a dysplastic or neoplastic disorder.

In a further embodiment, there is provided a method for treating a pathological growth of tissue in a patient, comprising administering to said patient an amount of at least one PTN antisense oligonucleotide sufficient to inhibit pleiotropin synthesis in said patient.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the cDNA sequence (SEQ ID NO:1) for human pleiotrophin.

FIGURE 2 depicts the location of the antisense primers within the cDNA sequence for human pleiotrophin (SEQ ID NO:2).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention involves methods for the inhibition of the synthesis of pleiotrophin, thus providing a therapeutic regimen for the treatment of neoplasias and dysplasias. The invention is based on the use of antisense oligonucleotides which anneal to pleiotrophin-specific single-stranded RNA, and which thereby inhibit production of pleiotrophin. Inhibition of pleiotrophin synthesis represses the corresponding

growth-stimulating activity and alleviates neoplastic and dysplastic conditions associated with PTN.

In accordance with the present invention oligonucleotides are provided that are designed to be hybridize to portions of the mRNA coding for pleiotrophin, thereby disrupting the functions of these RNA's.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the antisense oligonucleotides of the invention in combination with a pharmaceutically acceptable sterile vehicle, as described in Remingtons's Pharmaceutical Sciences; Drug Receptors and Receptor Theory, 18th ed., Mack Publishing Co., Easton, PA (1990).

Antisense technology offers a very specific and potent means of inhibition of this gene product. See Stein and Chang, *Science* 261:1004-12 (1993). Antisense oligonucleotides ("antisense oligos") are typically short sequences of DNA, usually 10-50 bases in length, that are complementary to specific regions of a corresponding target mRNA. Hybridization of antisense oligos to their target transcripts is highly specific as a result of complementary base pairing. Hybridization of antisense oligos is affected by such parameters as length, chemical modification and secondary structure of the transcript which can influence oligo access to the target site. See Stein *et al*, *Cancer Research* 48:2659 (1988).

In selecting the preferred length for a given oligo, a balance must be struck to gain the most favorable characteristics. Shorter oligos such as 10-to 15-mers, while offering higher cell penetration, have lower gene specificity. In contrast, while longer oligos of 20-30 bases offer better specificity, they show decreased uptake kinetics into cells. See Stein *et al.*, PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDE ANALOGUES in "Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression" Cohen, ed. McMillan Press, London (1988). Accessibility to mRNA target sequences also is of

importance and, therefore, loop-forming regions in targeted mRNAs offer promising targets.

In this disclosure the term "oligonucleotide" encompasses both oligomeric nucleic acid moieties of the type found in nature, such as the deoxyribonucleotide and ribonucleotide structures of DNA and RNA, and man-made analogues which are capable of binding to nucleic acids found in nature. The oligonucleotides of the present invention can be based upon ribonucleotide or deoxyribonucleotide monomers linked by phosphodiester bonds, or by analogues linked by methyl phosphonate, phosphorothioate, or other bonds. They may also comprise monomer moieties which have altered base structures or other modifications, but which still retain the ability to bind to naturally occurring DNA and RNA structures. Such oligonucleotides may be prepared by methods well-known in the art, for instance using commercially available machines and reagents available from Perkin-Elmer/Applied Biosystems (Foster City, CA).

Phosphodiester-linked oligonucleotides are particularly susceptible to the action of nucleases in serum or inside cells, and therefore in a preferred embodiment the oligonucleotides of the present invention are phosphorothioate or methyl phosphonate-linked analogues, which have been shown to be nuclease-resistant. See Stein *et al.* (1993), *supra*. Persons of ordinary skill in this art will be able to select other linkages for use in the invention. These modifications also may be designed to improve the cellular uptake and stability of the oligos. Ghosh *et al.*, *Anti-Cancer Drug Design* 7:1 (1992).

In another embodiment of the invention the antisense oligonucleotide is an RNA molecule produced by transfection of the target cell with an expression construct. The RNA molecule thus produced is chosen to hybridize to pleiotrophin mRNA, thus inhibiting translation of the mRNA and inhibiting pleiotrophin synthesis.

Hybridization of the oligos with mRNA targets can inhibit expression of corresponding gene products by multiple mechanisms. In "translation arrest," the interaction of oligos with target mRNA blocks the action of the ribosomal complex and, hence, prevents translation of the messenger RNA into protein. Haeuptle *et al.*, *Nucl. Acids. Res.* 14:1427 (1986). In the case of phosphodiester or phosphorothioate DNA oligos, intracellular RNase H can digest the targeted RNA sequence once it has hybridized to the DNA oligomer. Walder and Walder, *Proc. Natl. Acad. Sci. USA* 85:5011 (1988). As a further mechanism of action, in "transcription arrest" it appears that some oligonucleotides can form "triplex," or triple-helical structures with double stranded genomic DNA containing the gene of interest, thus interfering with transcription by RNA polymerase. Giovannangeli *et al.*, *Proc. Natl. Acad. Sci.* 90:10013 (1993); Ebbinghaus *et al. J. Clin. Invest.* 92:2433 (1993).

In one preferred embodiment, PTN oligonucleotides are synthesized according to standard methodology. Phosphorothioate modified DNA oligonucleotides typically are synthesized on automated DNA synthesizers available from a variety of manufacturers. These instruments are capable of synthesizing nanomole amounts of oligonucleotides as long as 100 nucleotides. Shorter oligos synthesized by modern instruments are often suitable for use without further purification. If necessary, oligos may be purified by polyacrylamide gel electrophoresis or reverse phase chromatography. See Sambrook *et al.*, *MOLECULAR CLONING: A Laboratory Manual*, Vol. 2, Chapter 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Alternatively, PTN oligonucleotides in the form of antisense RNA may be expressed transiently in appropriate cells from standard DNA expression vectors. PTN DNA sequences can be cloned from standard plasmids into expression vectors, which expression vectors have

characteristics permitting higher levels of, or more efficient expression of the resident oligonucleotides. At a minimum, these constructs require a prokaryotic or eukaryotic promoter sequence which initiates transcription of the inserted DNA sequences. A preferred expression vector is one where the expression is inducible to high levels. This is accomplished by the addition of a regulatory region which provides increased transcription of downstream sequences in the appropriate host cell. See Sambrook *et al.*, Vol. 3, Chapter 16 (1989).

For example, PTN antisense expression vectors can be constructed using the polymerase chain reaction (PCR) to amplify appropriate fragments from single-stranded cDNA of plasmid pRc-PTN. Fang *et al.*, *J. Biol. Chem.* **267** 25889-25897 (1992). Figure 2 discloses nucleotide sequences of suitable oligonucleotide primers for the PCR reaction. Oligonucleotide synthesis and purification techniques are described in Sambrook *et al.* and Ausubel *et al.* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience 1987) (hereafter "Ausubel"), respectively. The PCR procedure is performed via well-known methodology. See, for example, Ausubel, and Bangham, "The Polymerase Chain Reaction: Getting Started," *in* PROTOCOLS IN HUMAN MOLECULAR GENETICS (Humana Press 1991). Moreover, PCR kits can be purchased from companies such as Stratagene Cloning Systems (La Jolla, CA) and Invitrogen (San Diego, CA).

The products of PCR are subcloned into cloning vectors. In this context, a "cloning vector" is a DNA molecule, such as a plasmid, cosmid or bacteriophage, that can replicate autonomously in a host prokaryotic cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and

selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance. Suitable cloning vectors are described by Sambrook *et al.*, Ausubel, and Brown (ed.), MOLECULAR BIOLOGY LABFAX (Academic Press 1991). Cloning vectors can be obtained, for example, from GIBCO/BRL (Gaithersburg, MD), Clontech Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI), Stratagene Cloning Systems (La Jolla, CA), Invitrogen (San Diego, CA), and the American Type Culture Collection (Rockville, MD).

Preferably, the PCR products are ligated into a "TA" cloning vector. Methods for generating PCR products with a thymidine or adenine overhang are well-known to those of skill in the art. See, for example, Ausubel at pages 15.7.1-15.7.6. Moreover, kits for performing TA cloning can be purchased from companies such as Invitrogen (San Diego, CA).

Cloned antisense fragments are amplified by transforming competent bacterial cells with a cloning vector and growing the bacterial host cells in the presence of the appropriate antibiotic. See, for example, Sambrook *et al.*, and Ausubel. PCR is then used to screen bacterial host cells for PTN antisense orientation clones. The use of PCR for bacterial host cells is described, for example, by Hofmann *et al.*, "Sequencing DNA Amplified Directly from a Bacterial Colony," in PCR PROTOCOLS: METHODS AND APPLICATIONS, White (ed.), pages 205-210 (Humana Press 1993), and by Cooper *et al.*, "PCR-Based Full-Length cDNA Cloning Utilizing the Universal-Adaptor/Specific DOS Primer-Pair Strategy," *Id.* at pages 305-316.

Cloned antisense fragments are cleaved from the cloning vector and inserted into an expression vector. For example, *Hind*III and *Xba*I can be used to cleave the antisense fragment from TA cloning vector pCR™-II (Invitrogen;San Diego, CA). Suitable expression vectors typically contain (1) prokaryotic DNA elements coding for

a bacterial origin of replication and an antibiotic resistance marker to provide for the amplification and selection of the expression vector in a bacterial host; (2) DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence.

For a mammalian host, the transcriptional and translational regulatory signals preferably are derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Molec. Appl. Genet.* **1**: 273 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* **31**: 355 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature* **290**: 304 (1981); the Rous sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* **79**: 6777 (1982)); and the cytomegalovirus promoter (Foecking *et al.*, *Gene* **45**: 101 (1980)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control fusion gene expression if the prokaryotic promoter is regulated by a eukaryotic promoter. Zhou *et al.*, *Mol. Cell. Biol.* **10**: 4529 (1990); Kaufman *et al.*, *Nucl. Acids Res.* **19**: 4485 (1991).

A suitable vector for expression in mammalian cells is the vector pRc/CMV (Invitrogen (San Diego, CA), which provides a high level of constitutive transcription from mammalian enhancer-promoter sequences. Cloned PTN

antisense vectors are amplified in bacterial host cells, isolated from the cells, and analyzed as described above.

Another possible method by which antisense sequences may be exploited is via gene therapy. Virus-like vectors, usually derived from retroviruses, may prove useful as vehicles for the importation and expression of antisense constructs in tumor cells. Generally, such vectors are non-replicative *in vivo*, precluding any unintended infection of non-target cells. In such cases, helper cell lines are provided which supply the missing replicative functions *in vitro*, thereby permitting amplification and packaging of the antisense vector. A further precaution against accidental infection of non-tumor cells involves the use of tumor cell-specific regulatory sequences. When under the control of such sequences, antisense constructs would not be expressed in normal tissues.

Two prior studies have explored the feasibility of using antisense oligonucleotides to inhibit the expression of a heparin binding growth factor. Kouhara *et al.*, *Oncogene* 9: 455-462 (1994); Morrison, *J. Biol. Chem.* 266: 728 (1991). Kouhara *et al.* showed that androgen-dependent growth of mouse mammary carcinoma cells (SC-3) is mediated through induction of androgen-induced, heparin binding growth factor (AIGF). An antisense 15-mer corresponding to the translation initiation site of AIGF was measured for its ability to interfere with androgen-induction of SC-3 cells. At concentrations of 5 μM, the antisense oligonucleotide effectively inhibited androgen-induced DNA synthesis. Morrison showed that antisense oligonucleotides targeted against basic fibroblast growth factor can inhibit growth of astrocytes in culture. Thus, the general feasibility of targeting tumor-related growth factors has been established.

Antisense oligonucleotides according to the present invention are derived from any portion of the open reading frame of the pleiotrophin cDNA. Preferably,

mRNA sequences (i) surrounding the translation initiation site and (ii) forming loop structures are targeted. Based upon the size of the human genome, statistical studies show that a DNA segment approximately 14-15 base pairs long will have a unique sequence in the genome. To ensure specificity of targeting pleiotrophin RNA, therefore, it is preferred that the antisense oligonucleotides are at least 14 nucleotides in length, and preferably 15 nucleotides in length. Thus, oligonucleotides contemplated by the present invention encompass nucleotides corresponding to positions 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 2-16, 3-17, *etc.* of the pleiotrophin cDNA sequence. All possible oligonucleotides are represented by nucleotides according to the formula n to n + x, where n is 1 to 1383 and x is 14, 15, 16, 17, 18 or 19.

Not every antisense oligo will provide a sufficient degree of inhibition or a sufficient level of specificity for the PTN target. Thus, it will be necessary to screen oligonucleotides to determine which have the proper antisense characteristics. There are several methods by which one can screen oligos for inhibition of PTN synthesis. For example, there are numerous cell lines in which the synthesis of PTN is elevated (*e.g.*, HS578T and MDA-MB231 breast cancer cell lines, T98G glioblastoma cells, 1205-LU and WM852 melanoma cell lines). The levels of PTN produced by these cells may be determined, for example, by radioimmune precipitation, Western blot, RIA or ELISA. Treatment of PTN-producing cells with effective antisense oligonucleotides will cause a decrease in PTN levels.

Alternatively, it is possible to assay for PTN activity directly. As mentioned above, cell lines are available which have elevated levels of PTN. These cells also are characterized by certain behavioral abnormalities such as soft agar colony formation. In particular, the increased proliferation of endothelial cells can be measured, and this is a useful *in vitro*

model for angiogenesis *in vivo*. Treatment of such cells with effective antisense oligos will result in the alteration of the cell's behavior and serve to identify useful oligos. Assays such as those described above serve as standard models for tumor growth in the body.

Antisense oligonucleotides can be tested for *in vivo* efficacy and safety in an animal model system. A preferred animal model is one in which the animal bears tumors as closely related as possible to those found in humans. In a preferred embodiment, the mouse is a nude, athymic mouse carrying explanted human tumor cells which will produce clinical symptoms analogous to those observed in human cancer. Such a mouse is a standard animal model used in the development of chemotherapeutic drugs. See, for example, Pitot, "Fundamentals of Oncology" 3rd ed., Marcel Dekker, Inc., New York, 1986, at 452.

Administration of an antisense oligonucleotide to a subject, either as a naked, synthetic oligo or as part of an expression vector, can be effected via any common route (oral, nasal, buccal, rectal, vaginal, or topical), or by subcutaneous, intramuscular, intraperitoneal, or intravenous injection. Pharmaceutical compositions of the present invention, however, are advantageously administered in the form of injectable compositions. A typical composition for such purpose comprises a pharmaceutically acceptable solvent or diluent and other suitable, physiologic compounds. For instance, the composition may contain oligonucleotide and about 10 mg of human serum albumin per milliliter of a phosphate buffer containing NaCl.

As much as 700 milligrams of antisense oligodeoxynucleotide has been administered intravenously to a patient over a course of 10 days (*i.e.*, 0.05 mg/kg/hour) without signs of toxicity. Sterling, "Systemic Antisense Treatment Reported," *Genetic Engineering News* **12**: 1, 28 (1992).

Other pharmaceutically acceptable excipients include non-aqueous or aqueous solutions and non-toxic compositions including salts, preservatives, buffers and the like. Examples of non-aqueous solutions are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous solutions include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art. A preferred pharmaceutical composition for topical administration is a dermal cream or transdermal patch.

Antisense oligonucleotides or antisense expression vectors may be administered by injection as an oily suspension. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides. Moreover, antisense oligonucleotides or vectors may be combined with a lipophilic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension also contains stabilizers.

An alternative formulation for the administration of antisense PTN oligonucleotides involves liposomes. Liposome encapsulation provides an alternative formulation for the administration of antisense PTN oligonucleotides and expression vectors. Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. See, generally, Bakker-Woudenberg *et al.*, *Eur. J. Clin.*

Microbiol. Infect. Dis. 12 (Suppl. 1): S61 (1993), and Kim, *Drugs* 46: 618 (1993). Liposomes are similar in composition to cellular membranes and as a result, liposomes can be administered safely and are biodegradable. Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and liposomes can vary in size with diameters ranging from 0.02 μm to greater than 10 μm. A variety of agents can be encapsulated in liposomes: hydrophobic agents partition in the bilayers and hydrophilic agents partition within the inner aqueous space(s). See, for example, Machy *et al.*, LIPOSOMES IN CELL BIOLOGY AND PHARMACOLOGY (John Libbey 1987), and Ostro *et al.*, *American J. Hosp. Pharm.* 46: 1576 (1989). Moreover, it is possible to control the therapeutic availability of the encapsulated agent by varying liposome size, the number of bilayers, lipid composition, as well as the charge and surface characteristics of the liposomes.

Liposomes can adsorb to virtually any type of cell and then slowly release the encapsulated agent. Alternatively, an absorbed liposome may be endocytosed by cells that are phagocytic. Endocytosis is followed by intralysosomal degradation of liposomal lipids and release of the encapsulated agents. Scherphof *et al.*, *Ann. N.Y. Acad. Sci.* 446: 368 (1985).

After intravenous administration, conventional liposomes are preferentially phagocytosed into the reticuloendothelial system. However, the reticuloendothelial system can be circumvented by several methods including saturation with large doses of liposome particles, or selective macrophage inactivation by pharmacological means. Claassen *et al.*, *Biochim. Biophys. Acta* 802: 428 (1984). In addition, incorporation of glycolipid- or polyethelene glycol-derivatised phospholipids into liposome membranes has been shown to result in a significantly reduced uptake by the reticuloendothelial system. Allen *et al.*, *Biochim. Biophys. Acta* 1068: 133 (1991); Allen *et al.*, *Biochim.*

Biohys. Acta **1150**: 9 (1993) These Stealth® liposomes have an increased circulation time and an improved targeting to tumors in animals. Woodle *et al.*, *Proc. Amer. Assoc. Cancer Res.* **33**: 2672 (1992). Human clinical trials are in progress, including Phase III clinical trials against Kaposi's sarcoma. Gregoriadis *et al.*, *Drugs* **45**: 15 (1993).

Antisense oligonucleotides and expression vectors can be encapsulated within liposomes using standard techniques. A variety of different liposome compositions and methods for synthesis are known to those of skill in the art. See, for example, U.S. Patent No. 4,844,904, U.S. Patent No. 5,000,959, U.S. Patent No. 4,863,740, and U.S. Patent No. 4,975,282, all of which are hereby incorporated by reference.

Liposomes can be prepared for targeting to particular cells or organs by varying phospholipid composition or by inserting receptors or ligands into the liposomes. For instance, antibodies specific to tumor associated antigens may be incorporated into liposomes, together with antisense oligonucleotides or expression vectors, to target the liposome more effectively to the tumor cells. See, for example, Zelphati *et al.*, *Antisense Research and Development* **3**: 323-338 (1993), describing the use "immunoliposomes" containing antisense oligonucleotides for human therapy.

In general, the dosage of administered liposome-encapsulated antisense oligonucleotides and vectors will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Dose ranges for particular formulations can be determined by using a suitable animal model.

Doses and routes of admininstration also will vary depending upon the type of pathological growth. Growths may be dysplastic, *i.e.*, abnormal tissue growth that is benign in character, such as retinopathies, arthritis, psoriasis, nevi and virally-induced

dysplasias. Growths may also be neoplastic, *i.e.*, associated with tumor formation and malignancy, such as melanoma, breast cancer, ovarian cancer, prostate cancer, glioblastoma, neuroblastoma and metastatic disease

EXAMPLE 1. **MATERIALS AND METHODS:**

Cell Lines. SW-13 cells (human adrenal carcinoma) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and used as target cells in anchorage independent colony formation in soft agar as described previously. Fang *et al.* (1992). SW-13/PTN cells are SW-13 cells stably transfected with a PTN-containing clone to overexpress human pleiotrophin, as previously described. Fang *et al.* (1992). SW-13/MK cells are SW-13 cells transfected to overexpress the human midkine gene. Sale *et al.*, manuscript in preparation (1994). All SW-13 cell lines were maintained in improved minimum essential medium (IMEM; Biofluids Inc., Rockville, MD) with 10% fetal bovine serum (FBS; Biofluids Inc.). Human metastatic melanoma cells (1205-LU) were a gift from Dr. M. Herlyn of the Wistar Institute, Philadelphia, PA and were maintained in media containing 80% keratinocyte serum free medium (KSFM; GIBCO/BRL, Bethesda, MD) and 20% Leibowitz medium (L-15; GIBCO/BRL) with 5% FBS and 1.5 mM $CaCl_2$. Human brain endothelial cells (huBEC) were isolated in primary culture from human cerebellum and maintained in 199E medium with 10% FBS. The huBECs were a gift of Dr. P. Costello of the Department of Neurosurgery, Georgetown University, Washington, D.C.

Oligonucleotides. Phosphorothioate modified DNA oligonucleotides were synthesized as either 15-mers (ptnAS1/SCR1) or 20-mers (ptnAS3/SCR3) using phosphoramidite backbone chemistry on a Milligen 8750 DNA synthesizer (Millipore, Bedford, MA). The first antisense oligo was complementary to the translation initiation codon and the second was complementary to a loop-forming region in the open reading frame of PTN. The sequences used are as follows: ptnAS1 (SEQ ID NO:1)

= 5'-GAGCCTGCATTTTTG-3'; ptnSCR1 (SEQ ID NO:2) = 5'-ATGCTTACGTTTGCG-3'; ptnAS3 (SEQ ID NO: 3) = 5'-CCAGTATGAAAATGAATGCC-3'; ptnSCR3 (SEQ ID NO:4) = 5'-CAAGACGATTCCATAGTGAA-3'. The oligos were solubilized in PBS before addition to cells and their concentrations verified by optical density (OD_{260}). To ascertain that the oligos were intact and full-length samples were end-labeled with ^{32}P, run on a polyacrylamide gel and autoradiographed.

ELISA Assays. Conditioned media was removed from the treated cells at the specified times and filtered through low protein binding membranes to remove debris before plating 100-200 µl at serial dilutions in 96-well plates (MaxiSorp; Nunc, Thomas Scientific, Swedesboro, NJ). After plating, the wells were allowed to dry. Alternatively, the conditioned media was concentrated and partially purified by heparin-affinity chromatography before plating. Wellstein et al., (1992).

Each well was washed three times with 200 µl of phosphate buffered saline-0.5% Tween-20 wash solution (PBS/Tween) before each step and four times before the final step. Each well was treated with 100-200 µl PBS/Tween with 1% BSA for one hour at room temperature to block non-specific binding before addition of 100 µl primary PTN antibody at 1:500 diultion (PTN-1 rabbit antisera raised by this laboratory; PTN-HBNF rabbit antisera, the gift of Dr. P. Böhlen, Lederle Laboratories, Pearl River, N.Y. In the midkine ELISA, the primary MK antibody (MK rabbit antisera raised by this laboratory) was diluted 1:1000. After one hour of incubation at 4°C, 100 µl of the secondary antibody (goat anti-rabbit IgG-alkaline phosphatase; Boehringer Mannheim, Indianapolis, IN) at 1:3000 dilution was added and incubated at 4°C for one hour. The final step was addition of 100 µl substrate solution (pNPP = para-nitrophenyl phosphate; GIBCO/BRL; PNPP at 1 mg/ml in 10 mM pH 9.5 diethanolamine with 0.5 mM $MgCl_2$) which was allowed to develop for 5-60 minutes, depending on the

protein concentrations. Absorbance was measured on a microplate reader (Molecular Devices, Sunnyvale CA) at 405 nm.

Soft Agar Assays and Coculture Experiments. Formation of colonies in soft agar by SW-13, SW-13/PTN or SW-13/MK cells was determined as described previously. Wellstein *et al.* (1990). In brief, 20,000 cells in 0.35% agar (Bactoagar; GIBCO/BRL) were layered on top of 1 ml of a solidified 0.6% agar layer in a 35 mm dish (Costar Corp. Cambridge). Material to be tested was filter-sterilized, 500 μl of which was added with the 800 μl top layer unless indicated otherwise. Growth media with 10% FBS was included in both layers. Colonies more than 60 μm in diameter were counted after 1-2 weeks of incubation at 37°C using an image analyzer.

Conditioned media from PTN-expressing cells was added to SW-13 cells. Antisense oligos, as indicated in the text, were added to SW-13/PTN or SW-13/MK cells. In other studies, coculture of PTN-expressing cells and SW-13 cells was studied in a similar manner, except that the PTN-expressing cells were first plated on the bottom of 35 mm dishes at densities of 1×10^3 to 5×10^3 cells per dish, followed by addition of the agar layers and SW-13 cells. During the interval before the addition of agar and SW-13 cells, the PTN-expressing cells were allowed to adhere to the plastic and were treated with antisense oligos for the specified time and concentration.

Endothelial Cell CoCulture. huBEC cells were plated at a density of 5×10^3 per well in the bottom of a six-well culture plate (Falcon, Becton Dickinson, Franklin Lakes, NJ). 1205-LU cells were plated at a density of 5×10^4 in 0.45 μm porosity cell culture inserts (Falcon) and treated for 72 hours with either antisense oligo, control oligo (scrambled sequence) or phosphate buffered saline (PBS) before transferring the inserts into the huBEC culture plates for continued coculture of one week. The huBEC cells were then detached with trypsin and counted with a particle

counter. Alternatively, conditioned media from oligo-treated 1205-LU cells was harvested and added to the huBECs in culture at different concentrations. Cell numbers were counted after six days.

Tumor Growth in Animals. Pre-confluent melanoma cells were pretreated with the specified concentration of either antisense oligo DNA, control oligo DNA or PBS for 72 hours. After this time period, cells were trypsinized from the treatment flasks and washed three times in melanoma media before collection. Cells were then injected subcutaneously (1 X 10^6 cells in 100 μl of media) into the flanks of athymic nude mice (NCr nu/nu; Harlan Sprague-Dawley, Indianapolis, IN) and the diameter of tumors measured every other day after tumor became visibile, as described previously. Fang *et al.* (1992).

EXAMPLE 2 - SELECTION OF TARGET SEQUENCES IN THE PTN TRANSCRIPT:

Two different approaches were used to select the regions in PTN to be targeted. One region was chosen based on the fact that targeting of antisense molecules to the translation initiation site of mRNA has been shown to inhibit the translation of its protein product. See Stein *et al.* (1988), *supra*. We selected one antisense sequence (ptnAS1, SEQ ID NO:1) in that region. The second approach took the predicted secondary structure of PTN mRNA into account. We searched the predicted secondary structure of the PTN mRNA for loop-forming regions within the open reading frame. Zuker *et al.* *Nucl. Acids Res.* 9: :133 (1981). The uniqueness of these sequences was screened by GENBANK comparison. Additionally, the antisense oligos were selected for higher G+C content at their ends to improve hybridization characteristics. Stein *et al, loc. cit.* (1989). The control oligos were chemically-identical, scrambled sequences (ptnSCR1 and ptnSCR3, SEQ ID NOS 2 and 4) of the respective antisense oligos. It was determined that these oligos did not have a significant antisense relationship to other regions in PTN or in other genes.

Sense oligos were avoided as control sequences due to the fact that they are usually chemically different from the respective antisense oligos in as much as they contain different compositions of nucleotides. Furthermore, computer generated PTN mRNA folding patterns predicted numerous stem formations of the molecule. Obviously, self-hybridization between complementary strands thus is likely and sense oligos also could act to specifically inhibit PTN by binding to the respective complementary sequence in PTN.

EXAMPLE 3 - **SPECIFIC INHIBITION OF PTN PRODUCTION IN SW-13/PTN CELLS:**

To determine whether specific antisense inhibition of PTN expression could be achieved, we used SW-13 cells that had been transfected with a PTN expression vector. These cells form colonies in agar as a result of PTN activity. Fang *et al.* (1992). In these experiments, SW-13/PTN cell colony formation was inhibited by 64% when cells were treated with 10 μM of the antisense oligo (ptnAS1, SEQ ID NO:1) for 48 hours prior to seeding in soft agar. Analogous treatment with an equivalent concentration of the scrambled control oligo (ptnSCR1, SEQ ID NO:2), or with the vehicle, did not inhibit the colony forming potential of SW-13/PTN cells. When 3 μM of oligo was used, no differences between treatment groups were observed. When SW-13/PTN cells were treated with a concentration of 30 μM ptnAS1 for 48 hours prior to seeding in agar, there was no increased reduction in colony formation over that observed with the 10 μM treatment. Treatment with 30 μM of ptnSCR1, however, also inhibited the colony forming potential of SW-13-PTN cells, presumably by non-sequence specific effects. The antisense inhibition of PTN protein secretion was confirmed by ELISA of the supernatants from the cells. To test the specificity of PTN antisense oligos, SW-13 cells that had been transfected with an MK expression vector (SW-13/MK cells) were treated with PTN antisense oligos and assayed the

supernatants assayed for colony stimulating activity. MK synthesis also was measured by ELISA. SW-13/MK cells have been shown to form colonies in soft agar due to MK expression. Sale *et al.* (1994). No inhibition of MK bioactivity or drop in secretion of MK into the SW-13/MK supernatants was detected. Thus, although MK is closely related to PTN (50% sequence identity), it appears that our PTN antisense oligos are very specific. From these data, it appears that a specific, dose-dependent inhibition of PTN production and secretion can be achieved *in vitro*.

EXAMPLE 4 - LACK OF GROWTH INHIBITION IN VITRO OF MELANOMA CELLS EXPRESSING PTN:

1205-LU melanoma cells constitutively express high levels of PTN and form tumors very aggressively. Hartmann *et al.*, manuscript in preparation (1994). These cells were used as a model cell line to determine whether PTN plays a role in melanoma growth. Previously, it has been shown that human melanoma cells express PTN mRNA, whereas human melanocytes do not. Fang *et al.* (1992). The following studies were conducted to determine whether 1205-LU cells require autocrine-acting PTN for their growth and colony formation or, if they use PTN exclusively for paracrine growth stimulation of surrounding tissues.

It was found that 1205-LU metastatic melanoma cells do not require PTN to form colonies in soft agar. More specifically, when these cells are treated with antisense oligos that inhibit PTN production, they form the same number of colonies with the same size as those cells treated with both the scrambled oligo and PBS. In addition to the colony formation assay, melanoma cell proliferation was measured for one week during treatment with either antisense oligos or scrambled oligos at concentrations up to 20 μM. Regardless of oligo treatment or dose, cells proliferated to more than 12 times their original density. This suggests that the oligos themselves were not toxic to the melanoma cells.

As described below, oligo treatment was effective at inhibiting PTN production in these cells. It was concluded, therefore, that PTN was not rate-limiting for *in vitro* proliferation of 1205-LU cells.

EXAMPLE 5 - EFFECTS OF ANTISENSE TREATMENT ON PTN SECRETED FROM MELANOMA CELLS AND SW-13 CELL COLONY FORMATION:

Different growth factors released from melanoma cells, *e.g.*, from the FGF family, may stimulate SW-13 as well as endothelial cells. Rodeck *et al.*, *Cancer Metastasis Rev.* 10: 89 (1991). Endothelial cells respond to molecules such as FGFs, PTN and TGF-α, whereas SW-13 cells respond to FGFs, PTN, as well as IL-1,24 but not TGF-α. Conditioned media was collected from each 24 hour antisense treatment interval and assayed for PTN using both (i) a PTN-specific ELISA and (ii) SW-13 and endothelial cell responses.

Inhibition was not detected in the first 24 hours of treatment, most likely due to the residual PTN protein that was not yet cleared. The 48 hour samples showed a specific, dose-dependent decrease in the amount of PTN secreted, as measured by ELISA. At 10 μM of the antisense oligo, there was a 50% inhibition of immunoreactive PTN protein in the conditioned media while, at 10 μM, the scrambled oligo did not show any inhibition. Higher doses, while only allowing modest increases in effect, appear to cause non-specific inhibition of PTN synthesis as evidenced by the control oligo treatment. From these data, a concentration of 10 μM was chosen for further experiments in which a single dose was used.

In parallel with the ELISA, conditioned media from 1205-LU cells treated with antisense oligos was added to SW-13 cells that were seeded in a soft agar. As in the ELISA, the 24 hour samples did not show any significant inhibition of colony formation. The 48 hour samples (data not shown) and, especially the 72 hour samples, showed decreased colony stimulation activity

with antisense oligo treatment, indicating that constitutive secretion of PTN was reduced. At 10 μM, there was a 40% inhibition of target cell colony formation by the antisense oligo, while the control, scrambled oligo did not show any inhibition.

The data were similar with a different set of antisense and scrambled oligos (ptnAS3 and ptnSCR3, SEQ ID NOS 3 and 4). The antisense treatment inhibited more than 40% of the colony formation when compared to the controls. It also should be noted that at 20 μM, ptnAS3 (SEQ ID NO:3) inhibition was 64%, while the corresponding ptnSCR3 (SEQ ID NO: 1) oligo did not show any inhibition. When compared with ptnAS1 (SEQ ID NO:1) and ptnSCR1 (SEQ ID NO:2), it appears that ptnAS3 provides greater specificity. These data were supported by the ELISA-determined amounts of PTN found in the 1205-LU conditioned media treated with different oligo treatments and measured at different sampling times.

The ability of 1205-LU cells to stimulate directly SW-13 cells in coculture using soft agar also was evaluated. SW-13 cells did not exhibit any significant colony formation when the feeder layer was left empty. The addition of 1205-LU cells in the feeder layer, however, stimulated colony formation. When the 1205-LU feeder cells were pretreated for 72 hours with 10 μM ptnAS3, there was more than 50% inhibition of stimulatory activity as determined by SW-13 colony formation. Pretreatment of 1205-LU cells for the same amount of time with 10 μM ptnSCR3 did not reduce the stimulatory activity, confirming the sequence specificity of ptnAS3-mediated inhibition. Furthermore, these data demonstrate the ability of secreted PTN protein from human melanoma cells to stimulate epithelial cell growth in a direct paracrine fashion.

EXAMPLE 6 - BIOASSAY OF THE SUPERNATANTS USING ENDOTHELIAL CELL PROLIFERATION:

Earlier, it was reported that PTN secreted from human tumor cells can stimulate endothelial cell growth

in culture. Fang *et al.* (1992). The present study examined the PTN activity in media condition d by antisense-treated 1205-LU cells. This media was used to treat endothelial cells in parallel with SW-13 cells. The huBECs which were treated with 72 hour-conditioned media samples showed significantly less proliferation than controls. In fact, antisense treatment suppressed PTN activity to that of background levels. The 1205-LU-conditioned media showed comparative levels of PTN immunoreactivity, as measured by ELISA (data not shown).

Additionally, 1205-LU cells were plated in coculture to directly stimulate target huBEC cells. The same results were observed as with conditioned media harvested from 1205-LU cells (data not shown). The huBEC cells cocultured with ptnAS1- and ptnAS3-treated 1205-LU cells showed less proliferation than huBEC cells cocultured with control 1205-LU cells. The endothelial cells cocultured with ptnAS3-treated 1205-LU cells showed an almost 50% inhibition of proliferation in one week compared to the control (PBS-treated) coculture. Endothelial cells from the ptnSCR3 group, however, showed no significant decrease in cell proliferation over the same time period. A corresponding ELISA confirmed the inhibition of PTN protein in the respective 1205-LU conditioned media (data not shown). Endothelial cells which were not cocultured with 1205-LU cells received no cross-feeding activity and were used to determine background proliferation. These data not only demonstrate the ability of PTN protein, secreted from human melanoma cells, to stimulate endothelial cell growth in a paracrine fashion, but also demonstrate the ability of antisense DNA to reverse that stimulation.

EXAMPLE 7 - INHIBITION OF MELANOMA TUMORIGENESIS IN NUDE MICE:

To further define the role of paracrine stimulation by PTN secreted from melanoma cells and PTN-induced formation of solid tumors, melanoma cells were treated with oligos and then injected subcutaneously

into athymic nude mice. 1205-LU cells were pretreated for 72 hours as described above. This metastatic melanoma cell line grows into detectable tumors within a few days at the local site of injection.

At four days after injection, the tumors were found to be at least 10 mm in diameter, regardless of the pretreatment. This is likely due to the fact that tumors can grow to a certain size without recruiting blood vessels for nutrient support. Folkman *et al. J. Biol. Chem.* 267: 10931 (1992). Diffusion of oxygen and other nutrients should penetrate and feed these cells during the first few days of growth and tumor formation.

Conversely, it appears that the tumor must recruit microvasculature and surrounding stroma in a paracrine fashion for its continued growth support past this early stage. This is evidenced by the average doubling of tumor size in the control (PBS or ptnSCR1 pretreated cells) by the end of the first week after injection. In contrast, the antisense (ptnAS1) pretreated cells showed only a modest increase of less than 10% growth in this same time period. By nine days post-injection, the control tumors approximately tripled in size, while the antisense pretreated tumors increased in size by less than 25%.

Due to the eventual depletion of oligo from the original cell mass and its dilution with each tumor cell replication, the antisense inhibitory effect on PTN secretion is expected to "wash out" over the first week of tumor growth. By two weeks post-injection, the antisense-pretreated tumors were indistinguishable from the control tumors in terms of size. Although this experiment demonstrates the importance of PTN in the formation of human metastatic melanoma tumors, it also suggests the need for continued antisense treatment. Primarily, the results of these studies confirm the notion that PTN is indeed a rate-limiting growth factor in human metastatic melanoma growth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GEORGETOWN UNIVERSITY

(ii) TITLE OF INVENTION: Antisense Oligonucleotides Of Pleiotrophin

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: TO BE ASSIGNED
(B) FILING DATE: 18 JULY 1995 (18.07.95)
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGCCTGCAT TTTTG 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGCTTACGT TTGCG 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCAGTATGAA AATGAATGCC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAAGACGATT CCATAGTGAA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1383 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGTAAATAA ACTTTAAAAA TGGCCTGAGT TAAGTGTATT AAAAAGAAGA AATAGTCGTA 60

AGATGGCAGT ATAAATTCAT CTCTGCTTTT AATAAGCTTC CCAATCAGCT CTCGAGTGCA 120

AAGCGCTCTC CCTCCCTCGC CCAGCCTTCG TCCTCCTGGC CCGCTCCTCT CATCCCTCCC 180

ATTCTCCATT TCCCTTCCGT TCCCTCCCTG TCAGGGCGTA ATTGAGTCAA AGGCAGGATC 240

AGGTTCCCCG CCTTCCAGTC CAAAAATCCC GCCAAGAGAG CCCCAGAGCA GAGGAAAATC 300

CAAAGTGGAG AGAGGGGAAG AAAGAGACCA GTGAGTCATC CGTCCAGAAG GCGGGGAGAG 360

CAGCAGCGGC CCAAGCAGGA GCTGCAGCGA GCCGGGTACC TGGACTCAGC GGTAGCAACC 420

TCGCCCCTTG CAACAAAGGC AGACTGAGCG CCAGAGAGGA CGTTTCCAAC TCAAAAATGC 480

AGGCTCAACA GTACCAGCAG CAGCGTCGAA AATTTGCAGC TGCCTTCTTG GCATTCATTT 540

TCATACTGGC AGCTGTGGAT ACTGCTGAAG CAGGGAAGAA AGAGAAACCA GAAAAAAAAG 600

TGAAGAAGTC TGACTGTGGA GAATGGCAGT GGAGTGTGTG TGTGCCCACC AGTGGAGACT 660

GTGGGCTGGG CACACGGGAG GGCACTCGGA CTGGAGCTGA GTGCAAGCAA ACCATGAAGA 720

CCCAGAGATG TAAGATCCCC TGCAACTGGA AGAAGCAATT TGGCGCGGAG TGCAAATACC 780

AGTTCCAGGC CTGGGGAGAA TGTGACCTGA ACACAGCCCT GAAGACCAGA ACTGGAAGTC 840

TGAAGCGAGC CCTGCACAAT GCCGAATGCC AGAAGACTGT CACCATCTCC AAGCCCTGTG 900

GCAAACTGAC CAAGCCCAAA CCTCAAGCAG AATCTAAGAA GAAGAAAAAG GAAGGCAAGA 960

AACAGGAGAA GATGCTGGAT TAAAAGATGT CACCTGTGGA ACATAAAAAG GACATCAGCA 1020

AACAGGATCA GTTAACTATT GCATTTATAT GTACCGTAGG CTTTGTATTC AAAAATTATC 1080

TATAGCTAAG TACACAATAA GCAAAAACAA CCAATTTGGG TTCTGCAGGT ACATAGAAGT 1140

TGCCAGCTTT TCTTGCCATC CTCGCCATTC GAATTTCAGT TCTGTACATC TGCCTATATT 1200

CCTTGTGATA GTGCTTTGCT TTTTCATAGA TAAGCTTCCT CCTTGCCTTT CGAAGCATCT 1260

TTTGGGCAAA CTTCTTTCTC AGGCGCTTGA TCTTCAGCTC TGCGAAATTC CTTCGCTTTT 1320

TCTTAAGGGT TTCTGGCACA GCAGGAACCT CCTTCTTCTT CTCTTCTACA CCCTCTATGT 1380

ACC 1383

What is claimed is:

1. A method of inhibiting pleiotrophin expression in a cell, comprising introducing into said cell at least one antisense oligonucleotide that binds to a segment of a single-stranded mRNA transcribed from a pleiotrophin gene, and that inhibits pleiotrophin synthesis in said cell.

2. A method according to claim 1, wherein said pleiotrophin is human pleiotrophin and said cell is a human cell.

3. A method according to claim 1, wherein said oligonucleotide is operably linked to a promoter which is active in said cell, which produces an RNA transcript which binds to a segment of a single-stranded mRNA transcribed from a pleiotrophin gene, and thereby inhibits pleiotrophin synthesis in said cell.

4. A method according to claim 3, wherein said oligonucleotide and said promoter are carried in an expression vector.

5. A method according to claim 1, wherein said oligonucleotide is introduced in a pharmaceutically acceptable solvent or diluent.

6. A method according to claim 1, wherein said oligonucleotide is introduced in a liposome.

7. A method according to claim 4, wherein said oligonucleotide is introduced in a liposome.

8. A method according to claim 1, wherein said oligonucleotide consists of nucleotides **n** to **n + x** of Figure 1 (SEQ ID NO:5), wherein **n** is an integer from 1 to 1383 and **x** is selected from the group of integers consisting of 14, 15,16, 17, 18 and 19.

9. A method according to claim 2, wherein said oligonucleotide is:

(a) an oligonucleotide that hybridizes under intracellular conditions to human pleiotrophin mRNA, wherein said hybridization of said oligonucleotide inhibits translation of said mRNA;

(b) an oligonucleotide selected from the group consisting of:

(i) an antisense oligonucleotide which selectively binds to the translation initiation site (AUG) of said mRNA

and

(ii) an antisense oligonucleotide which selectively binds to a conserved loop-forming region of said mRNA;

or

(c) an oligonucleotide selected from the group consisting of:

(i) 5' GAG CCT GCA TTT TTG 3' (SEQ ID NO:1)

and

(ii) 5' CCA GTA TGA AAA TGA ATG CC 3' (SEQ ID NO:3.

10. A composition comprising at least one antisense oligonucleotide that, when introduced into a host cell, binds to a segment of a single-stranded mRNA transcribed from a pleiotrophin gene, and that inhibits pleiotrophin synthesis in said cell.

11. An oligonucleotide according to claim 10, wherein said pleiotrophin is human pleiotrophin.

12. An oligonucleotide according to claim 10, wherein said oligonucleotide is operably linked to a promoter, which produces an RNA transcript that binds to a segment of a single-stranded mRNA transcribed from a pleiotrophin gene and thereby inhibits pleiotrophin synthesis in said cell.

13. An oligonucleotide according to claim 12, wherein said oligonucleotide and said promoter are carried in an expression vector.

14. An oligonucleotide according to claim 10, wherein said oligonucleotide consists of nucleotides n to n + x of Figure 1 (SEQ ID NO:5), wherein n is an integer from 1 to 1383 and x is selected from the group of integers consisting of 14, 15, 16, 17, 18 and 19.

15. An oligonucleotide according to claim 11, wherein said oligonucleotide hybridizes under intracellular conditions to human pleiotrophin mRNA.

16. An oligonucleotide according to claim 15, selected from the group consisting of:

(a) an antisense oligonucleotide that selectively binds to the translation initiation site of said mRNA;

and

(b) an antisense oligonucleotide that selectively binds to a conserved loop-forming region of said mRNA.

17. An oligonucleotide according to claim 16, selected from the group consisting of:

(a) 5' GAG CCT GCA TTT TTG 3' (SEQ ID NO:1);

and

(b) 5' CCA GTA TGA AAA TGA ATG CC 3' (SEQ ID NO:3).

18. A method for treating a pathological growth of tissue in a patient, comprising administering to said patient an amount of at least one composition as recited in claim 10 sufficient to inhibit pleiotropin synthesis in said patient.

19. The method of claim 18, wherein the pathological growth of tissue is selected from the group of neoplastic disorders and dysplastic disorders.

20. The method of claim 19, wherein the neoplastic disorder is selected from the group consisting of melanoma, breast cancer, ovarian cancer, prostate cancer, glioblastoma, neuroblastoma and metastatic disease.

21. The method of claim 19, wherein the dysplastic disorder is selected from the group consisting of retinopathies, arthritis, psoriasis, nevi and virally-induced dysplasias.

22. A pharmaceutically useful preparation comprising a composition as recited in claim 10 in a pharmaceutically acceptable sterile vehicle.

23. A method for treating a pathological growth of tissue, comprising the step of inhibiting expression of a pleiotrophin gene.

24. The method of claim 23, wherein the pathological growth of tissue is selected from the group consisting of neoplastic disorders and dysplastic disorders.

25. The method of claim 24, wherein the neoplastic disorder is selected from the group consisting of melanoma, breast cancer, ovarian cancer, prostate cancer, glioblastoma, neuroblastoma and metastatic disease.

26. The method of claim 24, wherein the dysplastic disorder is selected from the group consisting of retinopathies, arthritis, psoriasis, nevi and virally-induced dysplasias.

FIG. 1A

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   1  AAGTAAATAA ACTTTAAAAA TGGCCTGAGT TAAGTGTATT AAAAAGAAGA
  51  AATAGTCGTA AGATGGCAGT ATAAATTCAT CTCTGCTTTT AATAAGCTTC
 101  CCAATCAGCT CTCGAGTGCA AAGCGCTCTC CCTCCCTCGC CCAGCCTTCG
 151  TCCTCCTGGC CCGCTCCTCT CATCCCTCCC ATTCTCCATT TCCCTTCCGT
 201  TCCCTCCCTG TCAGGGCGTA ATTGAGTCAA AGGCAGGATC AGGTTCCCCG
 251  CCTTCCAGTC CAAAAATCCC GCCAAGAGAG CCCCAGAGCA GAGGAAAATC
 301  CAAAGTGGAG AGAGGGGAAG AAAGAGACCA GTGAGTCATC CGTCCAGAAG
 351  GCGGGGAGAG CAGCAGCGGC CCAAGCAGGA GCTGCAGCGA GCCGGGTACC
 401  TGGACTCAGC GGTAGCAACC TCGCCCCTTG CAACAAAGGC AGACTGAGCG
 451  CCAGAGAGGA CGTTTCCAAC TCAAAAATGC AGGCTCAACA GTACCAGCAG
 501  CAGCGTCGAA AATTTGCAGC TGCCTTCTTG GCATTCATTT TCATACTGGC
 551  AGCTGTGGAT ACTGCTGAAG CAGGGAAGAA AGAGAAACCA GAAAAAAAAG
 601  TGAAGAAGTC TGACTGTGGA GAATGGCAGT GGAGTGTGTG TGTGCCCACC
 651  AGTGGAGACT GTGGGCTGGG CACACGGGAG GGCACTCGGA CTGGAGCTGA
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FIG. 1B

701 GTGCAAGCAA ACCATGAAGA CCCAGAGATG TAAGATCCCC TGCAACTGGA

751 AGAAGCAATT TGGCGCGGAG TGCAAATACC AGTTCCAGGC CTGGGGAGAA

801 TGTGACCTGA ACACAGCCCT GAAGACCAGA ACTGGAAGTC TGAAGCGAGC

851 CCTGCACAAT GCCGAATGCC AGAAGACTGT CACCATCTCC AAGCCCTGTG

901 GCAAACTGAC CAAGCCCAAA CCTCAAGCAG AATCTAAGAA GAAGAAAAAG

951 GAAGGCAAGA AACAGGAGAA GATGCTGGAT TAAAAGATGT CACCTGTGGA

1001 ACATAAAAAG GACATCAGCA AACAGGATCA GTTAACTATT GCATTTATAT

1051 GTACCGTAGG CTTTGTATTC AAAAATTATC TATAGCTAAG TACACAATAA

1101 GCAAAAACAA CCAATTTGGG TTCTGCAGGT ACATAGAAGT TGCCAGCTTT

1151 TCTTGCCATC CTCGCCATTC GAATTTCAGT TCTGTACATC TGCCTATATT

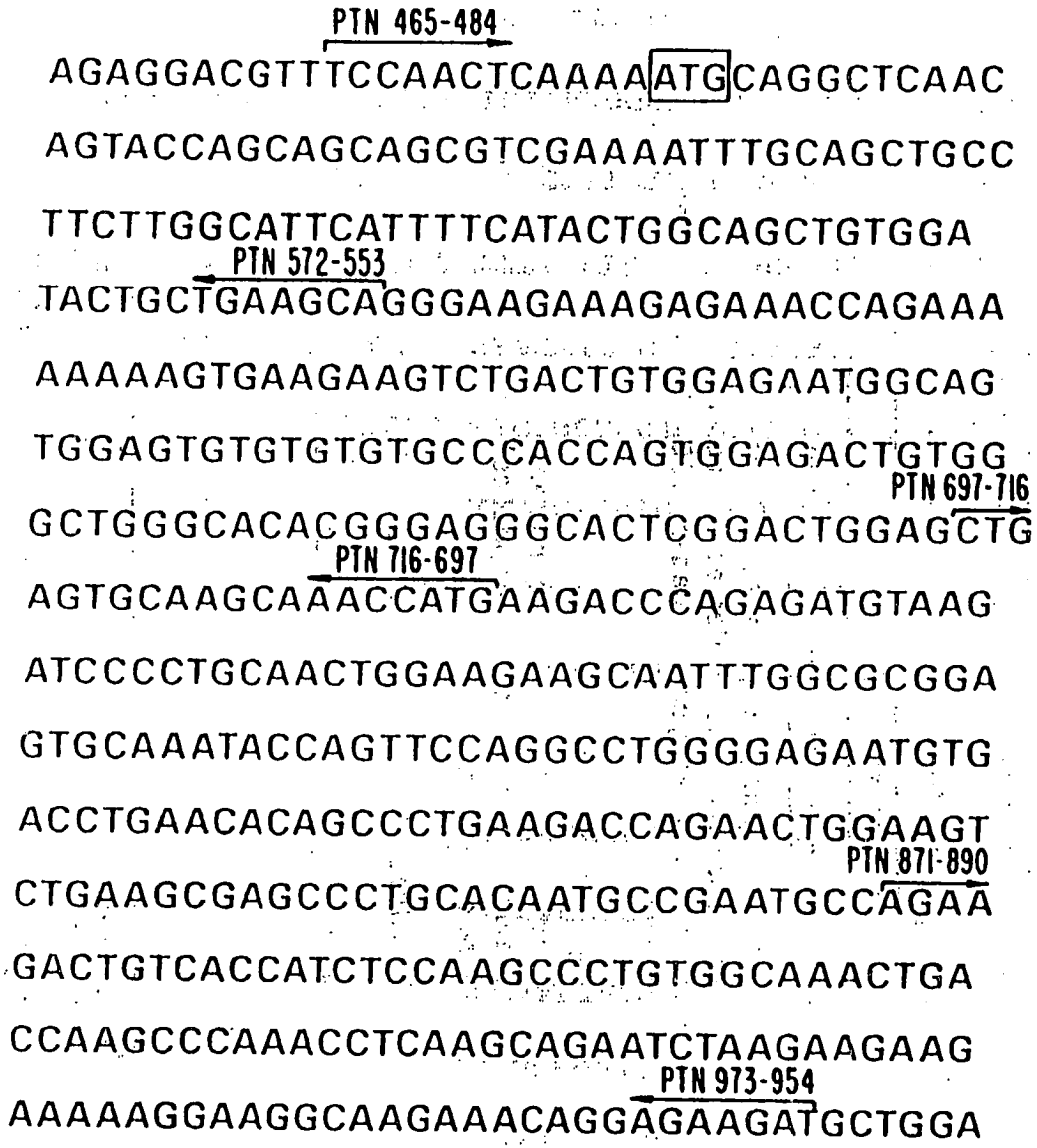
1201 CCTTGTGATA GTGCTTTGCT TTTTCATAGA TAAGCTTCCT CCTTGCCTTT

1251 CGAAGCATCT TTTGGGCAAA CTTCTTTCTC AGGCGCTTGA TCTTCAGCTC

1301 TGCGAAATTC CTTCGCTTTT TCTTAAGGGT TTCTGGCACA GCAGGAACCT

1351 CCTTCTTCTT CTCTTCTACA CCCTCTATGT ACC

FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08781

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/172.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline
search terms: pleiotrophin, heparin binding, growth factor, oligonucleotide, antisense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Science, Volume 261, issued August 1993, Stein et al., "Antisense Oligonucleotides as Therapeutic Agents - Is the Bullet Really Magical?", pages 1004-1011, see entire document.	1-26
A	Cancer Gene Therapy, Volume 1, Number 1, issued 1994, Tseng et al., "Antisense oligonucleotide technology in the development of cancer therapeutics", pages 65-71, see entire document.	1-26
A	S. CROOKE et al., "ANTISENSE RESEARCH AND APPLICATIONS", published 1993 by CRC Press (Boca Raton), pages 8-35, see entire document.	1-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 AUGUST 1995	Date of mailing of the international search report 28 NOV 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL LEWELL Telephone No. (703) 308-7307

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08781

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 267, Number 36, issued December 1992, Fang et al., "Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer", pages 25889-25897, see entire document.	1-26
Y, P	Journal of Biological Chemistry, Volume 269, Number 33, issued August 1994, Czubayko et al., "Ribozyme targeting elucidates a direct role of pleiotrophin in tumor growth", pages 21358-21363, see entire document.	1-26
Y	Biochemical and Biophysical Research Communications, Volume 187, Number 2, issued September 1992, Lai et al., "Structure of the human heparin-binding growth factor gene pleiotrophin", pages 1113-1122, see entire document.	1-26
A	Breast Cancer Research and Treatment, Volume 31, issued 1994, Wellstein, "Growth factor targeted and conventional therapy of breast cancer", pages 141-146, see pages 144-145.	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08781

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 31/70; C07H 21/02, 21/04; C12N 15/00, 5/00; C12P 19/34; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/44; 435/6, 91.1, 91.21, 172.1, 172.3, 240.2, 320.1; 536/23.1, 24.5